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TITLE: Prostate Cancer Immunotherapy by Targeting Dendritic Cells In Vivo Using Receptor-Specific Aptamer Conjugated to Prostate Stem Cell Antigen (PSCA)-Encoding RNA

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**14. ABSTRACT**

Dendritic cells (DCs), recognized as major antigen presenting cells, are uniquely equipped to initiate and regulate immune responses, making them a key target for developing new therapies. Although ex vivo DC therapy has shown promise, it is a customized, complex, patient-specific cell therapy that reduces its universal applicability for cancer. Directly targeting antigens to DCs in vivo will facilitate the development of an "off-the-shelf" tumor vaccine that circumvents the need for ex vivo DC loading. DCs express a number of specialized endocytic receptors of the C-type lectin family enabling capture of antigen by receptor-mediated endocytosis at very low concentrations. To deliver antigens to DCs in vivo, we developed artificial receptor ligands to the macrophage mannose receptor (MMR or CD206), an endocytic C-type lectin receptor expressed on DCs and macrophages. An RNA aptamer library comprised of 40 randomized nucleotides and modified with 2´Fluoro-modified pyrimidines for improved nuclease resistance was selected for affinity binding to MMR. A complex selection scheme with alternating rounds on both, recombinant human and murine MMR, as well as on cells engineered to express human MMR enabled us to identify cross-reactive aptamers that recognize MMR with high affinity. A truncated, minimal version of the identified aptamer with retained receptor-binding properties gets rapidly internalized into human monocyte-derived DCs in vitro.

**15. SUBJECT TERMS**
prostate cancer therapy, dendritic cell targeting in vivo, tumor antigen RNA

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INTRODUCTION:

The success of immunotherapy depends on the induction of effective primary immune responses to treat existing cancers and the generation of subsequent memory responses to prevent recurrence at a new site. The nature and magnitude of immune responses generated are influenced by the way an antigen is presented to the immune system. Dendritic cells (DC), recognized as major antigen presenting cells, are uniquely equipped to initiate and regulate immune responses, making them a key target for developing new therapies. Immunizing with antigen-loaded DC is a powerful method of inducing CD4+ and CD8+ T cell responses and antibodies (Abs). Although *ex vivo* DC therapy has shown promise, it is a customized, complex, patient-specific cell therapy that reduces its universal applicability for cancer. Thus there is a critical need to develop strategies that circumvent the need for *ex vivo* DC loading. Directly targeting antigens to DC *in vivo* will facilitate the development of an "off-the-shelf" tumor vaccine, which will be accessible to large numbers of patients. Moreover, DC targeting within their natural environment is thought to improve vaccine efficacy.

DC express a number of specialized endocytic receptors of the C-type lectin family enabling capture of antigen by receptor-mediated endocytosis at very low concentrations. Studies exploiting the endocytic properties of DC receptors demonstrated that antigens conjugated to receptor targeting moieties, such as the natural receptor-ligands or receptor-specific antibodies, increased antigen capture and presentation 100-1000-fold as compared to soluble antigen. Moreover, receptor targeting facilitates cross-presentation to induce Ab and CD4+ T cell immunity as well as elicit MHC-class-I-restricted CD8+ or cytotoxic T lymphocytes (CTL), critical for cancer immunotherapy.

To deliver antigens to DC *in vivo*, we developed artificial receptor ligands to the macrophage mannose receptor (MMR or CD206), an endocytic C-type lectin receptor expressed on DC and macrophages. Herein, a RNA aptamer library comprised of 40 randomized nucleotides and modified with 2´Fluoro-modified pyrimidines for improved nuclease resistance was selected for affinity binding to MMR. A complex selection scheme with alternating rounds on both, recombinant human and murine MMR, as well as on cells engineered to express human MMR enabled us to identify cross-reactive aptamers that recognize MMR with high affinity. A truncated, minimal version of the identified lead-aptamer that retains receptor-binding properties gets rapidly internalized into human monocyte-derived DC *in vitro*. Currently we are testing aptamer-tumor antigen chimeras and aptamer-decorated nanoparticles to deliver model tumor antigens to DC *in vitro* and *in vivo* and investigate their capability to elicit an antigen-specific immune response.

BODY:

Milestone #1: Get Institutional IACUC- (animal work) and IRB- (human work) approval. (1-4 months)
Human Protocol IRB# Pro00021695
Title: Leukapharesis of normal donors for *in vitro* immune assays
Expiration Date: 2/10/2012

IACUC# A360-09-12
Title: Prostate cancer immunotherapy by targeting dendritic cells *in vivo* using receptor-specific aptamer conjugated to prostate stem cell antigen (PSCA)-encoding RNA.
Expiration: 12/16/2012

**TASK 1: Generate and characterize aptamers that bind endocytic receptors expressed on murine and human DC. (1-12 months)**

Milestone 2: Generate aptamers for the study. (1-9 months)

1. Using 12 rounds of SELEX (systematic evolution of ligands by exponential enrichment), we identified RNA aptamers that bind the endocytic macrophage mannose receptor (MMR) receptors expressed on murine and human DC with high affinity and excellent specificity (Figure 1). The SELEX procedure ("toggle-SELEX") was designed to enable the identification of aptamers that cross-react with the extracellular domains of both, murine and human MMR (mMMR and hMMR), which share 83%

homology.

2. The identified lead aptamer, #14 (length: 71 nucleotides [nt]), was truncated to a 49nt version (#14A) with retained MMR-binding characteristics (Figure 2A and 2C), which allows efficient chemical synthesis. The introduction of two point mutations within a loop region of #14A resulted in a non-binding aptamer version, #14A_mut1, without altering the predicted secondary structure (Figure 2B and 2C). This aptamer serves as negative control in current and future experiments.

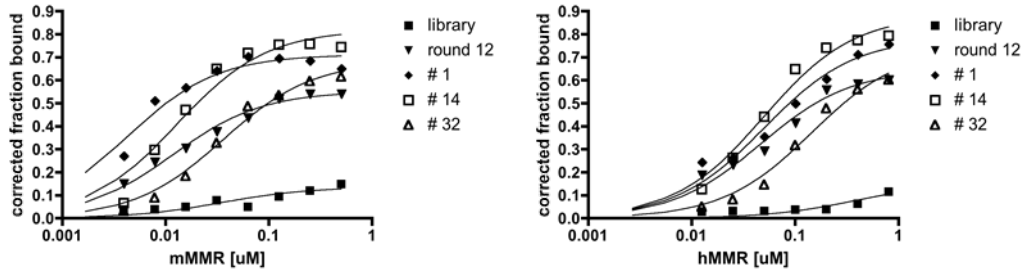


**Fig.1: 12 rounds of toggle-SELEX yielded aptamers that bind to both murine and human MMR (mMMR and hMMR) with high affinity**. γ-ATP-labeled RNA aptamers were incubated with serial dilutions of either recombinant mMMR (left panel) or hMMR (right panel) and binding monitored by nitrocellulose filter binding assays. The binding curves for the starting library (■), SELEX round 12 (▼), and three individual clones identified within the round 12 pool (# 1,♦; # 14,□; # 32,Δ) are shown.

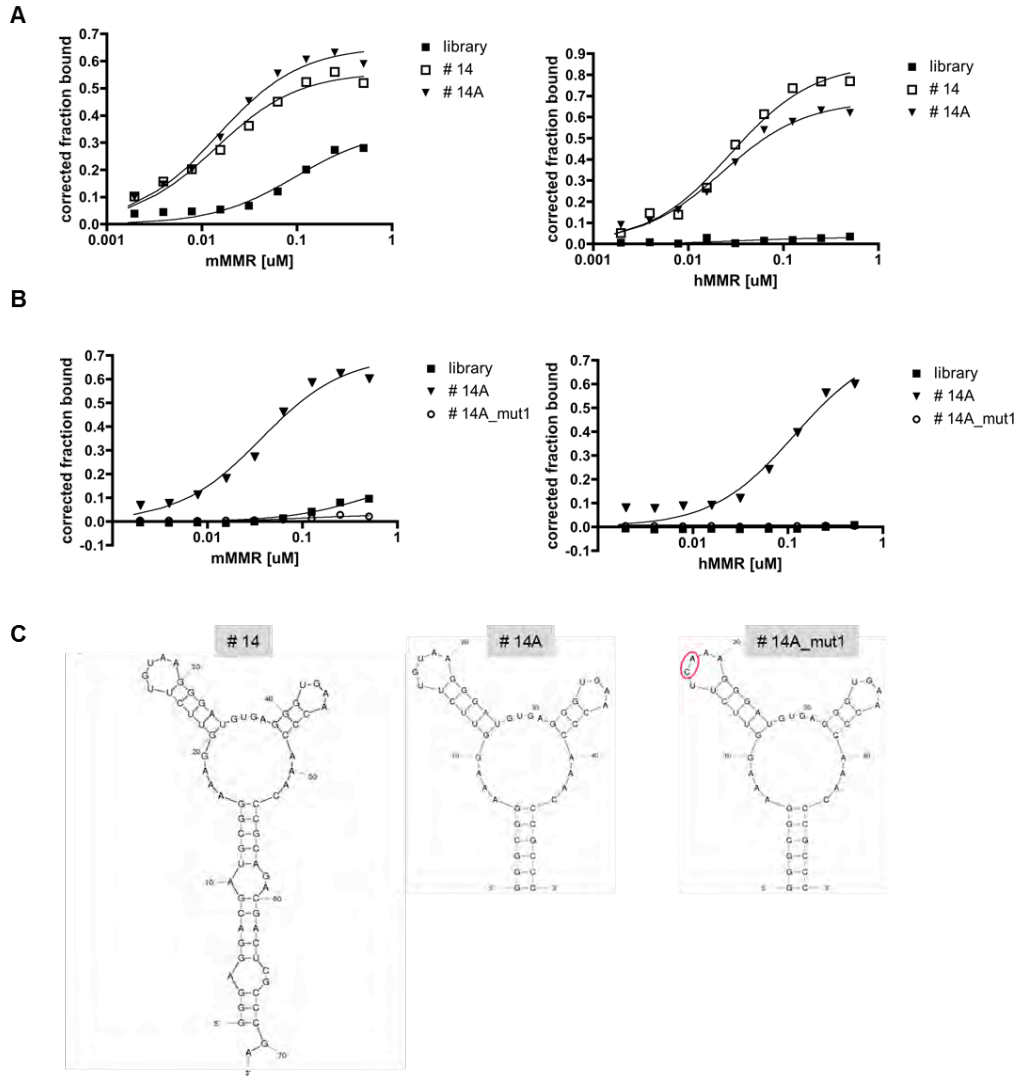

**Fig. 2: Characterization of aptamer #14 and its derivatives**. (**A**) Filter binding assays (as in Fig.1) were used to determine the mMMR (left panel)- and hMMR (right panel)-binding characteristics of the parental aptamer #14 (□) and its truncated version #14A (▼), as compared to the starting library (■). The $K_d$ and Bmax for #14 are 14nM and 59% on mMMR, and 28nM and 85% on hMMR, respectively. The truncated version #14A shows similar binding properties with a $K_d$ and Bmax of 15nM and 65% on mMMR, and 23nM and 68% on hMMR, respectively. (**B**) The introduction of two point mutations in #14A_mut1 leads to a complete loss of binding to both mMMR (left panel) and hMMR (right panel). (**C**) Aptamer secondary structures for #14 (left), #14A (middle), and #14_mut1 (right), as suggested by the mfold program (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). The two point mutations that distinguish #14A and #14A_mut1 are highlighted (red circle).

Milestone 3: Characterize aptamers for DC uptake. (6-12 months)

1. Flow cytometry assays using fluorescent-labeled #14 confirmed that the aptamer recognizes MMR in its native confirmation expressed on the surface of monocyte-derived human DCs (moDC; Figure 3A). Maturation of moDCs using a cytokine cocktail (mDC) leads to reduced cell staining efficiency as compared to immature DCs (imDC), which agrees with reports that DCs down regulate antigen capturing receptors, such as MMR, upon maturation. Moreover, the cell staining profile of the truncate #14A on imDC was found to be indistinguishable from the parental full-length aptamer (Figure 3C).
2. We further investigated uptake of fluorescent-labeled #14A into imDC upon binding to surface expressed MMR, which is a prerequisite to our overall goal to deliver tumor-antigens to DC *in vivo*. Incubation of imDC with #14A at $4^0$C (which abrogates receptor internalization) results in efficient cell staining, but the signal is lost upon treatment with RNase (Figure 4, left panel). In contrast, the cell staining profile of #14A on imDC at $37^0$C is not affected by RNase treatment, which indicates efficient receptor-mediated uptake of the aptamer into subcellular compartments (Figure 4, right panel).
3. We currently characterize the subcellular localization of aptamers internalized into imDC in more detail using confocal microscopy.
4. The uptake of #14A into murine DCs has not been studied so far, since we could not detect prominent MMR expression on easily accessible murine cell types, such as bone marrow-derived DC, primary splenocytes, or murine DC-derived cell lines (e.g. JAWS II, DC2.4). However, we identified high levels of MMR expressed on ~ 50% of lymph node resident $CD11C^+$ cells (data not shown), which supports the assumption that assessing the effect of antigen targeting to MMR expressing DC in murine immunotherapy models, as suggested in our proposal, is feasible.

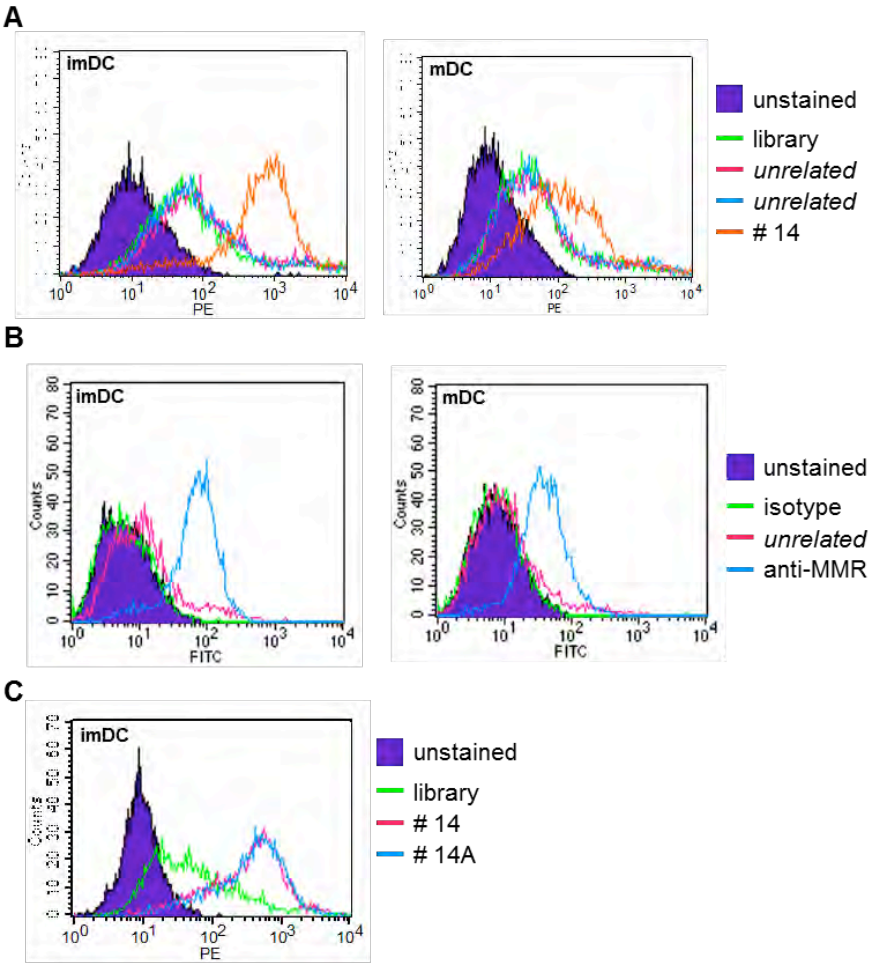


**Fig. 3: MMR expression on human DC as determined by flow cytometry using fluorescent labeled aptamers and antibodies**. (**A**) Human monocyte-derived imDC (left panel) and mDC (right panel) were stained with aptamers indirectly labeled with Phycoerythrin (PE). Herein, aptamers were transcribed with a 24nt extension at the 3' end and annealed to a complementary, biotinylated 25nt DNA oligonucleotide. Following capture of a Streptavidin:PE conjugate, the complexes were allowed to bind to the cells for 20 min at 37 C and cells analyzed by flow cytometry. Cell staining profiles are shown for the starting library, #14, and to other aptamers (*unrelated*). (**B**) Cell staining and flow cytometry analysis of the same cells as in (A) using commercial antibodies (as indicated) served as control. (**C**) imDC stained and analyzed as in (A) using #14 and the #14A truncate.

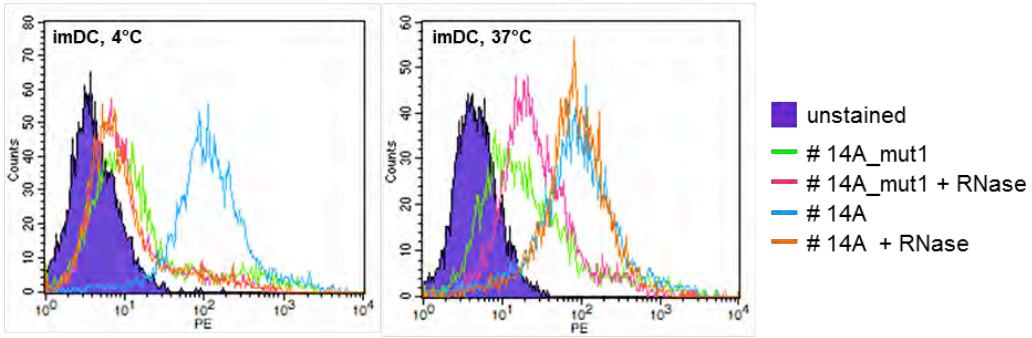


**Fig. 4: MMR receptor-specific specific internalization of aptamer #14A into imDC as monitored by flow cytometry**. Fluorescent labeled aptamers were allowed to bind to imDC in duplicate at either 4 C (left panel) or 37 C (right panel). After 20 min incubation, RNase was added to one of each duplicate to remove

cell surface bound aptamers. Cells were washed and analyzed by flow cytometry to assess the amount of internalized aptamers, which are protected from RNase-mediated degradation.

**TASK 2: Demonstrate that aptamer/mRNA chimeras can deliver tumor antigen to murine and human DC *in vitro*. (6-20 months)**

Milestone 4: Generate and optimize aptamer-mRNA chimeras. (6-18 months)

1. Since the induction of potent T cell responses positively correlates with the number antigenic peptides displayed in the context of major histocompatibility complex (MHC) class I and class II molecules, we compared the protein yield of mRNAs harboring a series of nucleobase modifications after transfection into different cell types. From all tested modifications, mRNAs incorporating either pseudouridine (ψ) or 5-methylcytidine (5mC) resulted in the highest translational output, with a 1.6- and 3.0-fold increase in protein yield, respectively, as compared to unmodified mRNA (Figure 5).
2. To establish aptamer-mRNA chimeras, we developed an optimized protocol to anneal #14A with a 25nt uridine extension at the 3'end to poly-adenylated mRNAs of choice (data not shown). #14A annealed to the poly-A tail of a 5mC-modified mRNA, radiolabeled at its 5'end, and encoding MART-1 is able to bind hMMR *in vitro* and efficiently tethers the mRNA to the protein (Figure 6).
3. We currently compare the intracellular localization and fate of internalized untargeted mRNA to MMR-targeted mRNA using confocal microscopy. Moreover, we investigate whether luciferase reporter-encoding mRNA targeted to MMR results in higher translational yield in imDC as compared to untargeted mRNA.
4. Besides our attempts to deliver mRNA to DC by means of aptamer #14A, we tested the capability of #14A to target protein antigens to DC. #14A-mediated protein delivery serves as internal quality control for the performance of our aptamer. As shown in Figure 7, we were able to specifically deliver a protein-complex consisting of chicken ovalbumin conjugated to IgG (molecular weight ~ 200kDa) to imDC using the MMR-targeting aptamer #14.

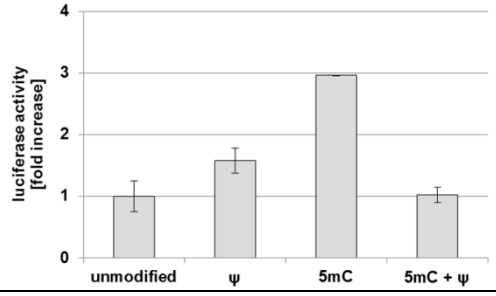


**Fig. 5: Comparison of the translational yield of mRNAs harboring different nucleobase modifications.** 10 µg of unmodified, pseudouridine modified (ψ), 5-methyl-cytosine modified (5mC), or 5mC + ψ modified mRNA encoding luciferase was transfected into RAW 267.4 cells using Lipofectamine 2000. The luciferase activity in cell lysates was determined after 24h and normalized to the activity of unmodified mRNA (mean of one experiment carried out in triplicate is shown).

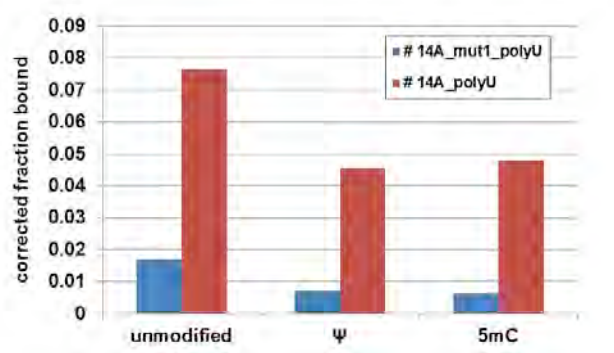


**Fig. 6: Aptamer-mediated binding of mRNA to hMMR protein *in vitro***. Aptamers #14A and #14A_mut1 were transcribed with a 25nt polyU extension at the 3' end and annealed to the polyA-tails of either unmodified, pseudouridine modified (ψ), or 5-methyl-cytosine modified (5mC) modified mRNA (γ-ATP-labeled at the 5' end). Binding of the resulting aptamer-mRNA chimeras to recombinant hMMR protein was determined by nitrocellulose filter binding.

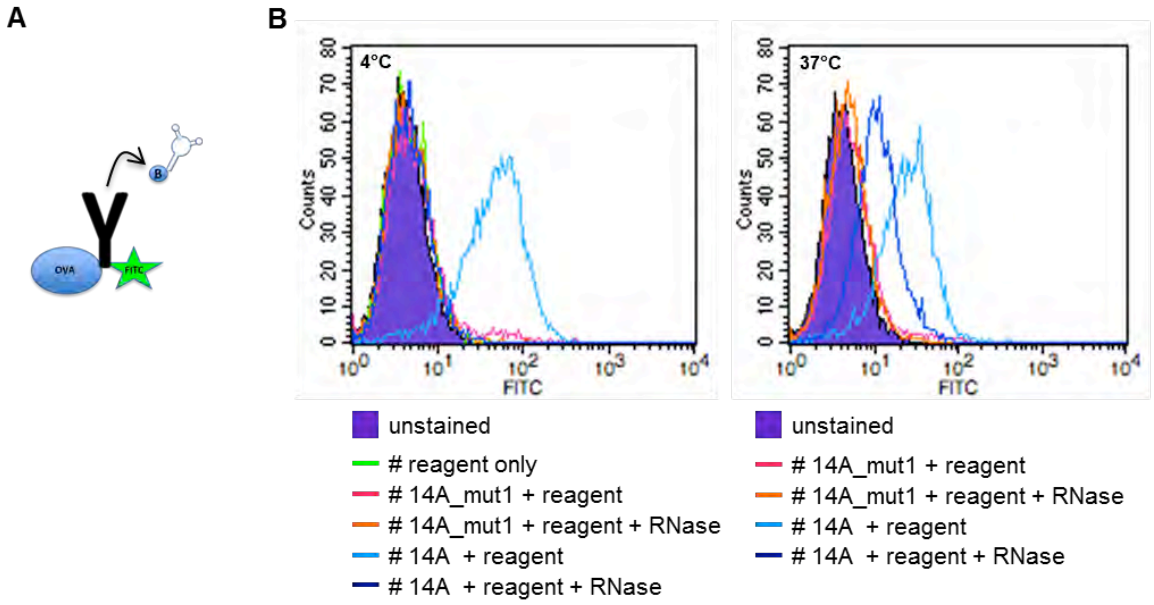


**Fig. 7: Aptamer-mediated delivery of protein to DC**. (**A**) Sketch of the reagents used in (B). The OVA-delivery reagent consists of a biotin-binding IgG-antibody (black) conjugated to full-length OVA protein (blue, egg-shaped symbol) and FITC-fluorophore (green, star-shaped symbol). This conjugate can be captured on 5' biotinylated aptamer (indicated by an arrow). (**B**) 5' biotinylated aptamers #14A_mut1 and #14A were allowed to bind imDC at 4 C for 20 min in quadruplicate. The cells were washed to remove unbound aptamers and further incubated with the OVA-delivery reagent (reagent) for capture on cell surface bound aptamers at 4 C. Two samples of each quadruplicate were further incubated at 4 C, while the remaining two samples were incubated at 37 C for 15 min to allow for receptor internalization. One of each duplicate samples incubated at either 4 C (left panel) or 37 C (right panel), respectively, was treated with RNase to remove cell surface bound aptamer/reagent complexes, the cells washed, and analyzed by flow cytometry. Fluorescent signals obtained in the presence of RNase represent protein complexes that have been internalized into the cell via the MMR-targeting aptamer #14A (dark blue population in the right panel).

KEY RESEARCH ACCOMPLISHMENTS:

Milestones achieved during the first year of funding as outlined in the Statement of Work (SOW):

Milestone #1: Obtained IACUC- (animal work) and IRB- (human work) protocol approval.

Milestone #2: Generated mouse and human MMR targeting aptamer for the study.

Milestone #3: Characterized MMR aptamer for DC uptake.
Milestone #4: Generated and optimized MMR aptamer-mRNA chimeras. (Preliminary data presented in this report, optimization is ongoing)

REPORTABLE OUTCOMES:
Data will be presented at 2 meetings:
Urban, J., D Boczkowski, K. Phua, K. Leong, B. Sullenger and S. Nair. Targeting tumor antigens to dendritic cells *in vivo* using receptor-specific aptamers conjugated to tumor antigen. Oligonucleotide Therapeutics Society meeting September 8-11, 2011, Copenhagen, Denmark

Urban, J., D Boczkowski, B. Sullenger and S. Nair. Targeting tumor antigens to dendritic cells *in vivo* using receptor-specific aptamers conjugated to tumor antigen. NCI Cancer Immunology and Immunotherapy meeting, September 22-23, 2011, Bethesda, Maryland.

We plan to submit our manuscript by January 2012.

CONCLUSIONS:
During our first year we have generated RNA aptamers, which bind both, human and murine MMR with high affinity and excellent specificity. We were able to truncate the lead aptamer to a 49nt version, which enables high yield chemical *de novo* synthesis. This aptamer is rapidly internalized into DC upon binding to MMR and allows the specific delivery of protein to DC *in vitro*. Thus, our accomplishments are on target as outlined in the SOW timeline. We will continue to achieve our objective to target prostate stem cell antigen (PSCA)-encoding mRNA vaccines to DC *in vivo* using receptor-specific aptamers.

REFERENCES:
NONE

APPENDICES:
NONE